

'Nitrification kinetics and microbial community dynamics of attached biofilm in wastewater treatment'

Adam Skoyles, Subba Rao Chaganti, Scott O. C. Mundle and Chris G. Weisener

ABSTRACT

A comparative bench-scale and field site analysis of BioCord was conducted to investigate seasonal microbial community dynamics and its impact on nitrogen removal in wastewater. This was assessed using metabolite (NO_3^-) stable isotope analysis, high-throughput sequencing of the 16S rRNA gene, and RT-qPCR of key genes in biological treatment representing nitrification, anammox, and denitrification. Bench-scale experiments showed an increase in nitrifiers with increasing ammonia loading resulting in an ammonia removal efficiency up to $98 \pm 0.14\%$. Stable isotope analysis showed that $\delta^{15}\text{N}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ could be used in monitoring the efficiency of the enhanced biological nitrification. In the lagoon field trials, an increase in total nitrogen promoted three principle nitrifying genera (*Nitrosomonas*, *Nitrospira*, *Candidatus Nitrotoga*) and enhanced the expression of denitrification genes (*nirK*, *norB*, and *nosZ*). Further, anaerobic ammonia oxidizers were active within BioCord biofilm. Even at lower temperatures ($2\text{--}6^\circ\text{C}$) the nitrifying bacteria remained active on the BioCord.

Key words | BioCord, high-throughput sequencing, microbial community, nitrogen, stable isotopes, wastewater

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INTRODUCTION

Nutrient pollution of water bodies is a global environmental problem that has led to increasingly stricter regulations in wastewater treatment processes (Lyu *et al.* 2016). Elevated levels of nitrogen in treated wastewater can lead to substantial environmental damage including eutrophication, harmful algal blooms, and the creation of hypoxic zones (Howarth 2008). The global biogeochemical cycling of nitrogen is almost entirely controlled through oxidation-reduction reactions by microbes (Falkowski 1997). As a result, they are commonly used by municipalities in biological wastewater treatment to reduce effluent nitrogen concentrations. Lagoon wastewater treatment plants (WWTP) are a commonly used treatment option for rural communities because they are less expensive and require more land than mechanical plants. However, nitrogen removal is less reliable in temperate regions due to the colder seasonal temperatures inhibiting microbial activity, which lagoon treatment systems rely on (Hurse & Connor 1999). Improvements to lagoon treatment systems in temperate environments are therefore required to effectively remove nitrogen, most importantly ammonia, year-round.

One proposed improvement to lagoon systems for effective nitrogen removal is an attached biofilm technology known as BioCord (Gan *et al.* 2018). BioCord provides a high surface area ring of polymer threads that encourages natural biofilm development. It has been successfully used to sustain a microbial community which reduces nutrient levels of effluent waters in various settings including laboratory, river and drainage ditch environments (Yuan *et al.* 2012; Tian *et al.* 2017; Zhou *et al.* 2018). However, there has not been any published research into using BioCord bio-reactors to upgrade a full size lagoon WWTP. Previous studies have also not characterized the active RNA-based BioCord microbial community, which is crucial to relating specific microbes to observed chemical changes.

It is widely known that nitrogen removal by microbes in wastewater has been accomplished through several metabolic processes including nitrification, denitrification, and anaerobic ammonium oxidation (anammox) (Munch *et al.* 1996; Strous *et al.* 1997; Schmidt *et al.* 2003). Nitrification is a two-step process where ammonium (NH_4^+) is first oxidized to nitrite (NO_2^-), which is then oxidized into nitrate

(NO₃⁻). The first step is completed by ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) and the second step is finished by nitrite-oxidizing bacteria (NOB). The exception is the recent discovery of some species from the *Nitrospira* genus that contain the genes for both steps and are known as complete ammonia oxidizing (comammox) bacteria (Daims et al. 2015). The more commonly found AOB in WWTPs are the genera *Nitrosomonas* and *Nitrosospira*, while the more common NOB are the genera *Nitrospira*, *Nitrobacter*, and the more recently discovered *Candidatus Nitrotoga* (Siripong & Rittmann 2007; Lucker et al. 2015; Cydzik-Kwiatkowska & Zielińska 2016). Denitrification is the process of reducing NO₃⁻ to molecular gaseous nitrogen (N₂) with intermediates of NO₂⁻, nitric oxide (NO), and nitrous oxide (N₂O). In order of reduction from NO₃⁻, these microbial processes are controlled by the enzymes nar, nir, nor, and nos, respectively. Anammox, the most recently discovered pathway for nitrogen removal, is the reaction of NH₄⁺ and NO₂⁻ to produce N₂ through a multistep process by a unique subgroup of *Planctomycetes* bacteria (Mulder et al. 1995; Kuenen 2008). Tracking the specific metabolic processes and their kinetics in addition to microbial dynamics is critical to predict accurate functionality of the system.

Compound specific isotope analysis of NO₃⁻ ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) has been used to investigate nitrogen cycling by measuring relative changes in isotopic fractionation caused by the increase in energy required to break the bond between heavy isotopes (Buchwald & Casciotti 2010). This approach has been important in quantifying kinetic effects of a changing environment on nitrification to optimize treatment strategies (Boshers et al. 2019). The isotopic fractionation effect of nitrification is relatively large but has been observed to vary significantly depending on the microbial community (Casciotti et al. 2003). Combining NO₃⁻ stable isotope analysis with a microbial community assessment will help to determine which microbes are responsible for the observed kinetic differences due to enhanced biological treatment.

Molecular methods used to assess microbial communities in wastewater treatment include targeted amplicon sequencing of the 16S rRNA gene and quantitative polymerase chain reaction (qPCR) (Harms et al. 2003; Yapsakli et al. 2011; De Sotto et al. 2018). High-throughput sequencing (HTS) of the amplified 16S rRNA gene is one of the most widely used tools to assess the bacterial population and recently it has also been shown to have a good representation of nitrifying microbial guilds (Diwan et al. 2018). In comparison, qPCR is a more effective tool for quantifying

a specific pathway or bacterial group (e.g. AOB). The total microbial community can often be significantly different from the active community (Yu & Zhang 2012). In order to represent the active community, the extracted RNA was used in this study.

The goal of this study was to assess the microbial community to identify key members of that community and determine the metabolic function of nitrogen removal within the BioCord. This will provide important information to effectively design and operate attached-biofilm systems with the goal of promoting specific microbes in temperate environments. A bench-scale experiment was designed to isolate the signatures of ammonia removal using BioCord and was compared to scaled observations in a field setting consisting of a full-scale lagoon WWTP. This study offers the first novel insight into the active microbial community dynamics of BioCord in both bench-scale and field settings and will be assessed using water chemistry, stable isotopes, HTS of the 16S rRNA region, and RT-qPCR.

METHODS

Sampling, bench-scale design, and field site description

Bench-scale design and sampling

The bench-scale experiments consisted of a continuous flow-through system designed to measure the chemical effect of BioCord treatment and the microbial community response. The flow-through system used two parallel sets of 3.5 L volume receiving vessels: one containing a 30 cm length suspended BioCord and one without any BioCord (Figure 1(a)). The influent water and BioCord were obtained from the fourth lagoon of the field system in October 2017. The water was preserved at 4 °C to slow down biological activity but reached room temperature during aerated treatment (e.g. 20 °C) upon entry into the vessels. The hydraulic retention time (HRT) of the system was maintained at six days. The system was set up in duplicate and the experiment was run over 28 days. At the end of a seven-day period, ammonium chloride (NH₄Cl) was added to the influent water thus increasing the chemical gradient each week (4.01, 10.3, and 28.2 mg/L NH₃-N). During each incremental seven-day cycle, 50 mL of water was collected from the effluent for chemical analysis. Samples of BioCord were also collected at each time point in duplicate and preserved at -80 °C until RNA extraction.

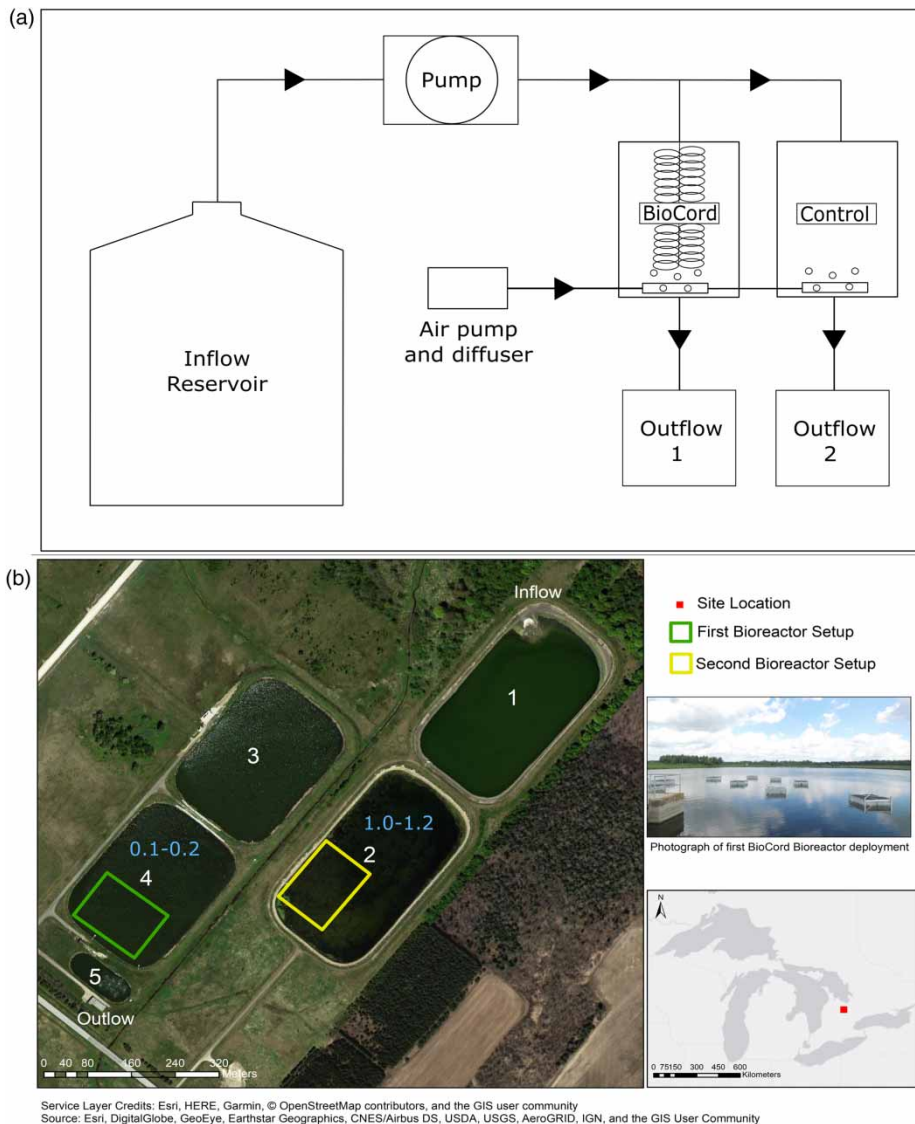


Figure 1 | (a) Bench-scale design overview; (b) field site overview of lagoon wastewater treatment facility in Dundalk, Ontario, Canada. Corresponding ammonia concentration ranges during times of BioCord sample collection shown for lagoons two and four in blue (mg/L). Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/wst.2020.170>.

Field site description and sampling

The wastewater treatment lagoons are a municipal treatment system in Dundalk, Ontario, Canada (44°09'12.0"N 80°23'07.0"W) and consist of five lagoons in series shown in Figure 1(b). Wastewater enters directly into lagoon one and is released from lagoon five into the upper Grand River watershed, in Ontario. The residence time of each lagoon varies between 30 and 60 days. In July 2016, 10 BioCord bioreactors were deployed surrounding the outflow pipe in lagoon four (L4). To test the microbial response to an increased nitrogen gradient, the rafts were removed from L4 and 10 new rafts were deployed near the outflow pipe of

lagoon two (L2) in July of 2017. In both locations, an aeration system was setup underneath all the rafts to keep the system well oxygenated year-round and to prevent ice formation around the rafts during the colder seasons. Water samples were collected weekly from the source inflow of the lagoon WWTP and within each lagoon. Biofilm samples were collected in the bioreactor deployments in L4 and L2 in late October of 2016 and 2017, respectively. Samples were also collected in late November of 2016 and early December of 2017 when water temperatures significantly decreased from 16.9 to 1.6 °C and 15.0 to 6.0 °C, respectively. Samples were collected by selectively cutting off portions of the BioCord into 5 mL cryotubes and then

preserved in liquid nitrogen. They were transferred to a -80°C freezer until RNA extraction. The number of samples collected is shown in Table A1 (Supplementary Material). An assessment using ANOSIM of the top 500 operational taxonomic units (OTUs) based on Bray-Curtis with 9999 permutations and sequential Bonferroni correction showed no significant differences between samples collected from eight rafts in 2016. As a result, a representative subset of two rafts was selected for sample collection in 2017.

Water chemistry and stable isotope analysis

Water chemistry and isotope analysis in the bench-scale experiment

In the bench-scale experiment, the collected outflow was used to measure the concentrations of nitrate, ammonia as nitrogen, and the pH. The pH was measured using a pH meter and the nitrate and ammonia as nitrogen were measured using ORION Aquafast II AC2007 and AC2012 respectively with an ORION AQ4000 spectrophotometer. Using $0.2\ \mu\text{m}$ nylon filters, 25 mL of filtered water was collected from each of the two systems at the beginning of the bench-scale experiment and every following seven days for the remainder of the study. The filtered samples were kept frozen at -20°C before stable isotope analysis. The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of NO_3^- in the outflow were measured as the inflow concentration of $\text{NH}_3\text{-N}$ was increased to determine potential fractionation effects of BioCord. The net nitrification rate (NNR) was calculated using the following equation (k = rate constant):

$$k = \frac{(C_o - C)}{t}$$

where C and C_o represent the concentrations of $\text{NH}_3\text{-N}$ at time t and initial, respectively. The $^{15}\text{N}/^{14}\text{N}$ ratio of the NH_4Cl that was added to the inflow was analyzed in triplicate with an elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) coupled with a continuous-flow isotope ratio mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). The ratios for $^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$ of dissolved NO_3^- in the effluent were analyzed using the denitrifier method (Sigman *et al.* 2001; Casciotti *et al.* 2002;). Briefly, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of dissolved NO_3^- was measured using an HP Agilent 6890 Gas Chromatograph with a PreCon[®] device interfaced to a Finnigan Mat DELTAplus XL mass spectrometer, with a precision of 0.5 ‰ for $\delta^{15}\text{N}$ and 1.0 ‰ for $\delta^{18}\text{O}$. The isotopic ratios

were then calculated using:

$$\delta^{15}\text{N} \text{ or } \delta^{18}\text{O} (\text{‰}) = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \times 1,000$$

where $R = ^{15}\text{N}/^{14}\text{N}$ or $^{18}\text{O}/^{16}\text{O}$. The standards used for N and O were atmospheric N_2 and standard mean ocean water (SMOW), respectively. Estimates for the $\delta^{15}\text{N}$ isotope enrichment effect ($^{15}\epsilon$) were calculated based on the following relationship where $f = [\text{NH}_4^+]_{\text{final}}/[\text{NH}_4^+]_{\text{initial}}$ (Mariotti *et al.* 1981):

$$\delta^{15}\text{N} - \text{NO}_3^-_{\text{final}} = \delta^{15}\text{N} - \text{NH}_4^+_{\text{initial}} - ^{15}\epsilon \left[\frac{f \times \ln(f)}{(1-f)} \right]$$

Water chemistry analysis for WWTP lagoons

Water samples were collected over the four times points from each of the lagoon outflows and the WWTP inflow. The measured parameters included water temperature, pH, dissolved oxygen (DO), chemical oxygen demand (COD), (soluble) biochemical oxygen demand (sBOD/BOD), alkalinity, total/volatile suspended solids (TSS/VSS), nitrogen speciation (NO_3^- , NO_2^- , $\text{NH}_3\text{-N}$, total N (TN), total Kjeldahl nitrogen (TKN)), and phosphorus (P/ortho-P) (Table A2).

RNA extraction, high-throughput sequencing, and RT-qPCR

RNA extraction and targeted amplicon sequencing of the 16S rRNA gene

The total RNA was extracted from all samples (two BioCord loops in each extraction) using RNeasy Powersoil Total RNA isolation kits following the instructions of the manufacturer. The Applied Biosystems High Capacity cDNA Reverse Transcription Kit was then used on the extracted RNA. The synthesized cDNA from all the samples was amplified targeting the V5-V6 region of the 16S rRNA gene (all primers shown in Table A3). The first PCR₍₁₎, purification using Agencourt AMPure XP, and the second PCR₍₂₎ for barcoding was done following the methods outlined in Falk *et al.* (2018). The PCR₍₂₎ products showed similar concentrations in agarose gel images and were all pooled together as a result. The pooled samples were run on a 1% agarose gel in triplicate then excised and extracted using QIAquick Gel Extraction Kit. The Agilent 2100 Bioanalyzer was used to check the final sample library to

test the quality and determine appropriate dilution for sequencing. Samples were then sequenced using the Ion Torrent PGM Next Gen Sequencer (Environmental Genomics Facility, University of Windsor).

The sequence reads have been deposited at the NCBI Sequence Read Archive under the accession PRJNA543086. The obtained 16S rRNA amplified dataset was quality filtered (Q20), barcodes trimmed, and demultiplexed using QIIME 1.9 (Caporaso *et al.* 2010). Samples containing read totals <5,000 were filtered out from downstream analysis. Chimera sequences were identified and filtered using VSEARCH (Rognes *et al.* 2016). Sequences were clustered into OTUs with open reference picking using the uclust algorithm with a 97% similarity threshold (Edgar 2010). Samples were then filtered to remove singleton and doubleton OTUs followed by taxonomy assignment using uclust with SILVA as the reference database (Quast *et al.* 2013). The OTUs were normalized using cumulative sum-scaling (CSS) for taxonomy analysis (Paulson *et al.* 2013). The unnormalized OTU table was normalized using the average of 10 rarefactions with a minimum of 5,000 sequences per sample to calculate alpha-diversity. To test the similarity of the microbial community between treatments in the bench-scale study and timepoints in the field study, ANOSIM of the top 500 OTUs based on Bray-Curtis with 9,999 permutations was used with sequential Bonferroni correction. One-way analysis of variance (ANOVA) of square root transformed relative abundance data was used to determine if there were any statistical differences of specific taxa between samples followed by Tukey post-Noc test to determine which samples were specifically different ($p < 0.05$). Canonical correspondence analysis (CCA) was used to observe dominant trends between chemical parameters and cDNA based taxonomic abundances. Statistical analysis was completed using Past version 3 (Hammer *et al.* 2001).

RT-qPCR

To take a more quantitative approach in community assessment, seven different targets were selected for quantitative amplification shown in Table A3. The targets were chosen to measure the amount of active AOB, active NOB, active anammox bacteria (AMX), and the expression of denitrification activity (*nirK*, *nosZ*, and *norB*). The amplification of the 16S rRNA gene was used as a reference gene for all targets to more accurately compare amplification between samples. The RT-qPCR reactions had a 10 μ L reaction volume containing 5 μ L Applied Biosystems PowerUp SYBR Green master mix, 0.4 μ L F primer (10 μ M), 0.4 μ L R primer

(10 μ M), 1 μ L cDNA Sample, and 3.2 μ L ddH₂O. All samples were run on the Quantstudio 12 K Flex Real-Time PCR System (Environmental Genomics Facility, University of Windsor). The thermocycler profile used followed 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR efficiencies were determined and corrected for using LinRegPCR and the RT-qPCR data were analyzed using the starting concentration (N_o) ratios (Ruijter *et al.* 2009). The N_o ratios were calculated by dividing the target N_o value by the N_o value for the 16S rRNA reference gene and averaging all individual sample values for that timepoint. One-way ANOVA of logarithmic transformed gene abundance data was used to determine if there were any statistically significant differences between samples for each gene followed by Tukey post-Noc test to determine which samples were specifically different ($p < 0.05$).

RESULTS AND DISCUSSION

Bench-scale water chemistry, nitrification kinetics, and stable isotope analysis

The influent and effluent pH remained moderately alkaline (7.8–8.4) over the course of the experiment. The initial influent NH₃-N concentration was 0.07 mg/L and was followed by three successive treatments. After seven days, in the first treatment 4.01 mg/L NH₃-N was added followed by 10.3 mg/L after 14 days and 28.2 mg/L after 21 days. In the BioCord system, the NH₃-N concentrations significantly decreased after each treatment to 0.14 ± 0.01 mg/L after 14 days, 0.13 ± 0.01 mg/L after 21 days, and 16.2 ± 0.8 mg/L after 28 days. Compared to the control system which contained no biocord, the NH₃-N concentration in the effluent was 4.02 ± 0.01 mg/L, 8.4 ± 0.4 mg/L, and 22.2 ± 1.1 mg/L after 14, 21 and 28 days of treatment, respectively. The resulting NH₃-N removal efficiencies ranged from $43 \pm 2.8\%$ to $98 \pm 0.14\%$ in the BioCord system and 0% to $21 \pm 3.9\%$ in the control system. The NO₃⁻ concentrations entering the systems showed a gradual increase after 21 days from <0.1 mg/L to 0.87 mg/L. The effluent from the BioCord system after 14, 21, and 28 days of treatment contained 5.3 ± 0.32 mg/L, 12.6 ± 0.06 mg/L, and 17.9 ± 1.2 mg/L of NO₃⁻, respectively. In comparison, the control system effluent NO₃⁻ concentrations were 0.3 ± 0.01 mg/L, 6.0 ± 0.2 mg/L, and 16.3 ± 0.7 mg/L, respectively. The contrasting increase in NO₃⁻ and decrease in NH₃-N suggests that nitrification occurred in both

systems with BioCord resulting in significantly enhanced nitrification. The calculated NNR was significantly higher in the BioCord system and increased as $\text{NH}_3\text{-N}$ concentration increased in the influent. As the $\text{NH}_3\text{-N}$ increased, the NNR ranged from 0.55 to 1.71 mg/L/day and 0 to 0.85 mg/L/day in the BioCord and control systems, respectively. The biofilm used in this study was perhaps limited with respect to surface area and was likely unable to effectively reduce $\text{NH}_3\text{-N}$ in the final treatment. Additionally, the bench-scale system may have required a longer residence time for the higher concentration gradient or required more oxygen stimulus (Ødegaard 2006). Although the NO_3^- concentrations were similar in both systems after 28 days, the $\text{NH}_3\text{-N}$ removal efficiency was double in the BioCord system, suggesting incomplete nitrification in the control system. This could also suggest that heterotrophic assimilation or anaerobic nitrogen removal processes (i.e. denitrification and anammox) were occurring within the biofilm. This was suggested in a previous study where anaerobic denitrifier DNA was observed in the BioCord community but the activity was not confirmed (Yuan et al. 2012).

Baseline NO_3^- concentrations were <0.1 mg/L for the control and 0.95 ± 0.04 mg/L for the BioCord system. The corresponding baseline isotope values for $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ were 20.6‰, 32.8‰, and 23.4‰, -3.5 ‰ for the control and BioCord, respectively. The increase in concentration of NO_3^- and distinct $\delta^{18}\text{O}_{\text{NO}_3}$ values for the control and BioCord suggest that the biofilm associated with the BioCord stimulated NO_3^- production within the baseline system prior to amendments with $\text{NH}_3\text{-N}$. The change in $\delta^{18}\text{O}_{\text{NO}_3}$ values suggests that the BioCord pathway catalyzes

oxidation with the oxygen sourced from the water, where the oxygen isotope value reflects projected values for the inflow source water (Yang et al. 1996). The system treated with 4.01 mg/L $\text{NH}_3\text{-N}$, which resulted in an increased concentration of NO_3^- to 5.34 ± 0.32 mg/L in the BioCord system, showed a $\delta^{15}\text{N}_{\text{NO}_3}$ value of 16.6‰, and $\delta^{18}\text{O}_{\text{NO}_3}$ value of -8.5 ‰. In comparison, the control system NO_3^- concentration was 0.3 ± 0.01 mg/L with $\delta^{15}\text{N}_{\text{NO}_3}$ value 12.2‰, and $\delta^{18}\text{O}_{\text{NO}_3}$ value -22.4 ‰. The change in $\delta^{15}\text{N}_{\text{NO}_3}$ values were consistent with the ^{15}N -depletion anticipated for isotopic fractionation from the $\text{NH}_3\text{-N}$ amendment to the inflow ($\delta^{15}\text{N}_{\text{NH}_4}$ value = 9.9 ± 0.1 ‰). As the $\text{NH}_3\text{-N}$ amendments were increased in the bench-scale study with the addition of NH_4Cl , the BioCord and control systems showed different relationships for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values. The $\delta^{15}\text{N}_{\text{NO}_3}$ values decreased in both systems as NO_3^- concentrations increased but showed a linear relationship ($R^2 = 0.9904$, $p = 0.0048$) and logarithmic relationship ($R^2 = 0.9635$, $p = 0.0184$) in the BioCord and control systems, respectively (Figure 2). The logarithmic relationship in the control system is likely a result of lag time in nitrifier growth, which has been observed in other nitrification studies, and was the reason why negligible nitrification was observed in the first treatment of the control system (Boshers et al. 2019). The enhanced nitrification in the BioCord system did not show a lag time and resulted in relatively less fractionation of the $\delta^{15}\text{N}_{\text{NO}_3}$ values. A linear regression of $\delta^{15}\text{N}_{\text{NO}_3}$ values versus $[-f \times \ln f / (1-f)]$ for all samples in the BioCord and control where nitrification was observed resulted in an enrichment factor of $^{15}\epsilon = -17.8 \pm 4.1$ ‰ ($R^2 = 0.8621$, $p = 0.0227$), which is comparable to nitrification enrichment factors reported in

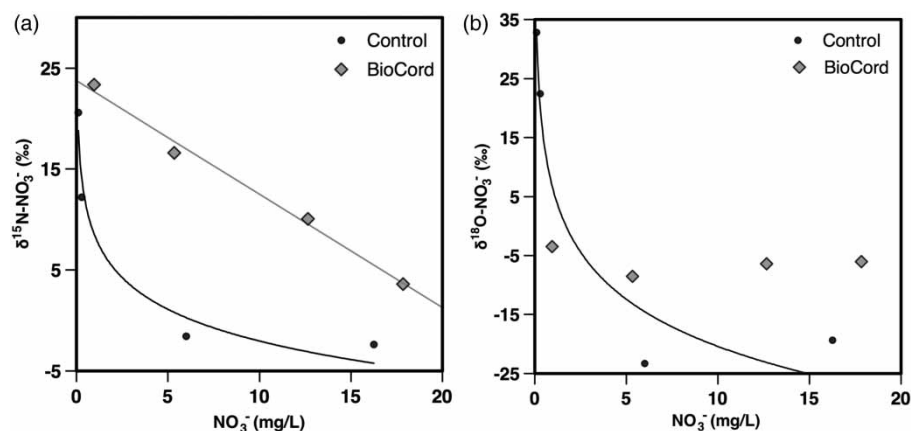


Figure 2 | Nitrate isotopic data in the bench-scale system throughout all treatments in the BioCord and control experiments. (a) The $\delta^{15}\text{N}_{\text{NO}_3}$ (‰) values versus the outflow NO_3^- concentrations (BioCord: $R^2 = 0.9904$, $p = 0.0048$ and control: $R^2 = 0.9635$, $p = 0.0184$); (b) $\delta^{18}\text{O}_{\text{NO}_3}$ (‰) values versus the outflow NO_3^- concentrations (control: $R^2 = 0.9495$, $p = 0.0256$).

other experiments (Casciotti et al. 2003). The $\delta^{18}\text{O}_{\text{NO}_3}$ values for the control system followed a similar logarithmic relationship ($R^2 = 0.9495$, $p = 0.0256$) as the $\delta^{15}\text{N}_{\text{NO}_3}$ values but the BioCord system only showed a slight change in $\delta^{18}\text{O}_{\text{NO}_3}$ values with increasing nitrate concentrations and remained close to the proposed $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values. The $\delta^{18}\text{O}_{\text{NO}_3}$ values have been shown to approach the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ value during nitrification. The deviation observed in the control system is attributed to the lag phase of growth and reduced NNR which increases both the NO_2^- equilibrium isotopic exchange of O with H_2O and the effect of kinetic O isotope fractionation (Buchwald & Casciotti 2010; Boshers et al. 2019). These observations suggest that the similarity of $\delta^{18}\text{O}_{\text{NO}_3}$ values to $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values could be a useful proxy for determining whether BioCord is impacting nitrification rates and thus could be used for optimization and efficiency monitoring. If the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{NO}_3}$ are analyzed before implementation of the treatment, then the nitrification rate changes could be approximated independently from the concentration in a WWTP based on the change in isotope value.

Although the NNR continued to increase in the last treatment with the BioCord (1.45 to 1.71 mg/L/day), and the $\delta^{18}\text{O}_{\text{NO}_3}$ did not change, the efficiency decreased significantly from $98 \pm 0.14\%$ to $43 \pm 2.8\%$. This suggests the system was still working normally but a longer residence time or an increase in surface area was required to successfully treat the high $\text{NH}_3\text{-N}$ levels. Based on the isotopic data, the BioCord was shown to accelerate oxidation by augmenting nitrification pathways in the system rather than alter the mechanism. The increasing NNR in the BioCord system suggests there could be substantial attachment and active growth of nitrifying organisms which was confirmed through HTS and RT-qPCR. Essential nutrients for these nitrifying organisms, such as P or trace metals, may have been limited, which could also have contributed to the decreased efficiency at higher loading.

Bacterial community dynamics based on high-throughput sequencing

Overview of bacterial communities within field and bench-scale environments

The microbial community structure and function of BioCord were investigated in both bench-scale experiments and full-scale lagoon WWTP field trials. A total of 16,488 OTUs were generated from 1,523,960 filtered sequence reads. To understand the effect of changing environmental conditions

(e.g. temperature) on species richness of the biofilm the chao1 alpha-diversity metric was compared using ANOVA followed by Tukey post-Noc test. The chao1 values showed no significant differences within the bench-scale samples but increased in lagoon samples. There was no observed difference between L2 samples, but richness significantly increased from October to November in L4 with colder temperatures ($p = 0.004$). Species richness was significantly increased in October L4 compared to October L2 ($p = 0.005$) and November L4 compared to December L2 ($p = 0.002$). This suggests the physicochemical differences between lagoons resulted in the active L2 BioCord community being less diverse than L4, which coincides with previous research showing an increase in diversity from beginning to end of a lagoon system (Mohn & Zhongtang 2001).

The similarity of microbial communities within the bench-scale and field experiments was assessed using ANOSIM. Results demonstrate there were no significant differences between the overall initial microbial community and the BioCord after each successive treatment in the bench-scale experiment. Within the field trials, there was no significant difference between the BioCord samples collected from L2 in October and December 2017. However, the samples collected from L4 in October and November 2016 were significantly different from each other and the L2 samples ($p = 0.0001$). To assess the BioCord community dynamics between environments, the OTUs from L4, L2, and bench-scale samples were respectively compiled and determined to be significantly different from each other ($p = 0.0001$).

To compare specific BioCord community differences between bench-scale and field sites, the average relative abundances of bacterial phyla were compiled for bench-scale experiments, L4, and L2 samples. All phyla that had greater relative abundance than 1% in the microbial community are shown in Figure 3. The most dominant phylum in the BioCord bacterial community was *Proteobacteria* followed by *Bacteroidetes*, with the most dominant *Proteobacteria* taxonomic classes being *Alphaproteobacteria* and *Betaproteobacteria*. *Deltaproteobacteria* and *Gamma-proteobacteria* were both present but significantly less abundant in comparison. *Actinobacteria* and *Acidobacteria*, were also dominant phyla throughout the BioCord community. *Cyanobacteria* was the third most abundant phylum in L4 but was not as dominant in L2 and bench-scale samples. In contrast, *Planctomycetes* and *Verrucomicrobia* were significantly less abundant in the bench-scale and L4 samples compared to L2. *Nitrospirae*, an important nitrifying phylum in wastewater treatment, was a minor community

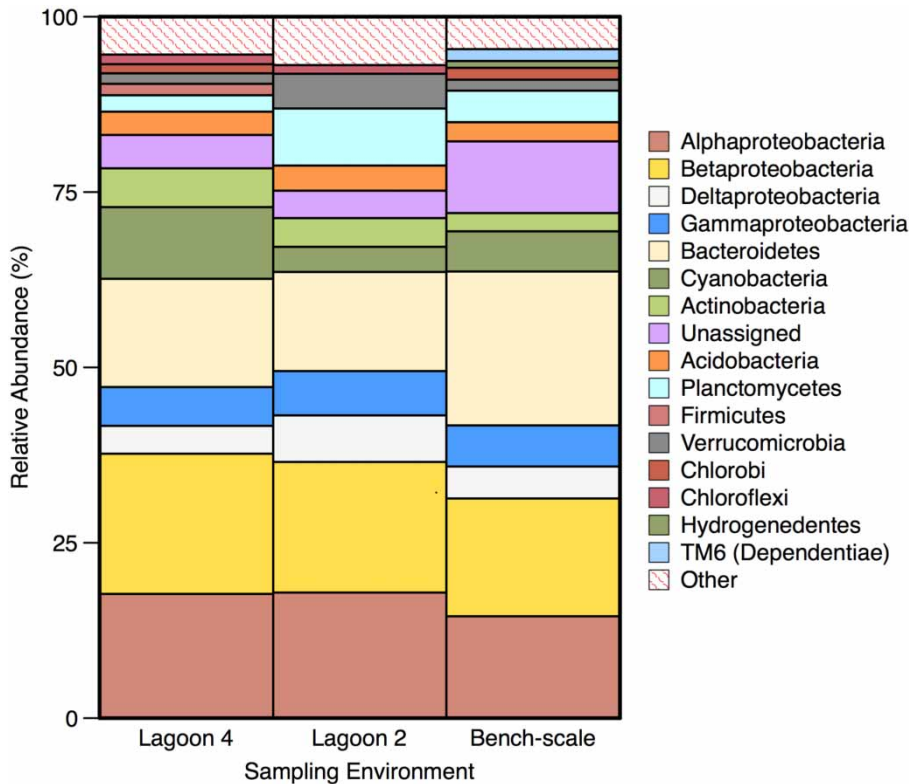


Figure 3 | Relative abundances greater than 1% within the bacterial phyla for the averages of samples within bench-scale, lagoon two and lagoon four. The total relative abundance for proteobacteria phyla is subdivided into taxonomic class. Lagoon four, lagoon two, and bench-scale samples are significantly different based on top 500 OTUs ANOSIM ($p = 0.0001$).

member representing 0.3%, 0.7%, and 0.6% in L4, L2, and bench-scale samples, respectively. Previous microbial characterizations of BioCord using extracted DNA found similar total community structures of bacteria phyla with the exception of *Planctomycetes*, which was not detected in bench-scale, and *Verrucomicrobia* which was determined to be significantly more dominant in an eco-ditch environment (Yuan et al. 2012; Zhou et al. 2018). Furthermore, this study found the *Proteobacterial* class to be largely dominated by *Alpha/Betaproteobacteria* while the previously mentioned studies determined *Betaproteobacteria* or *Alpha/Gamma-proteobacteria* to dominate.

Nitrifying bacterial community in the bench-scale experiment

Within the observed *Proteobacteria* and *Nitrospirae* phyla, the primary nitrifying genera were determined to be *Candidatus Nitrotoga*, *Nitrosomonas*, *Nitrospira*, and an uncultured genus of *Nitrosomonadaceae*, which have all been widely found in wastewater treatment (Cydzik-Kwiatkowska & Zielińska 2016). *Nitrospira* and *Nitrosomonas*

were detected in the previous bench-scale BioCord community study but *Nitrospira* and *Candidatus Nitrotoga* were not previously observed (Yuan et al. 2012). There was a consistent increase in the total relative abundance of nitrifiers in the bacterial community as $\text{NH}_3\text{-N}$ influent concentrations increased (Figure 4). Although both NOB, *Candidatus Nitrotoga* and *Nitrospira*, were present in all samples, *Nitrospira* was consistently the more dominant nitrite-oxidizer being 1.6 to 6.7 times more abundant. The relative abundance of *Nitrospira* did not increase after the first 4.01 mg/L $\text{NH}_3\text{-N}$ treatment but showed a significant increase after 10.3 and 28.2 mg/L $\text{NH}_3\text{-N}$ was added in the system ($p = 0.021$ and $p = 0.013$). The identified *Nitrosomonas* genus gradually increased to be the dominant AOB from 0.4 to 1.5 times abundance compared to the uncultured genus of *Nitrosomonadaceae*. Similar to *Nitrospira*, *Nitrosomonas* only increased significantly after the 10.3 mg/L $\text{NH}_3\text{-N}$ amendment ($p = 0.001$). However, it also continued to increase after 28.2 mg/L $\text{NH}_3\text{-N}$ was added and represented 3.3% of OTUs making it the second most abundant genus in the community ($p = 0.012$). Overall, the bench-scale conditions were relatively more favourable for

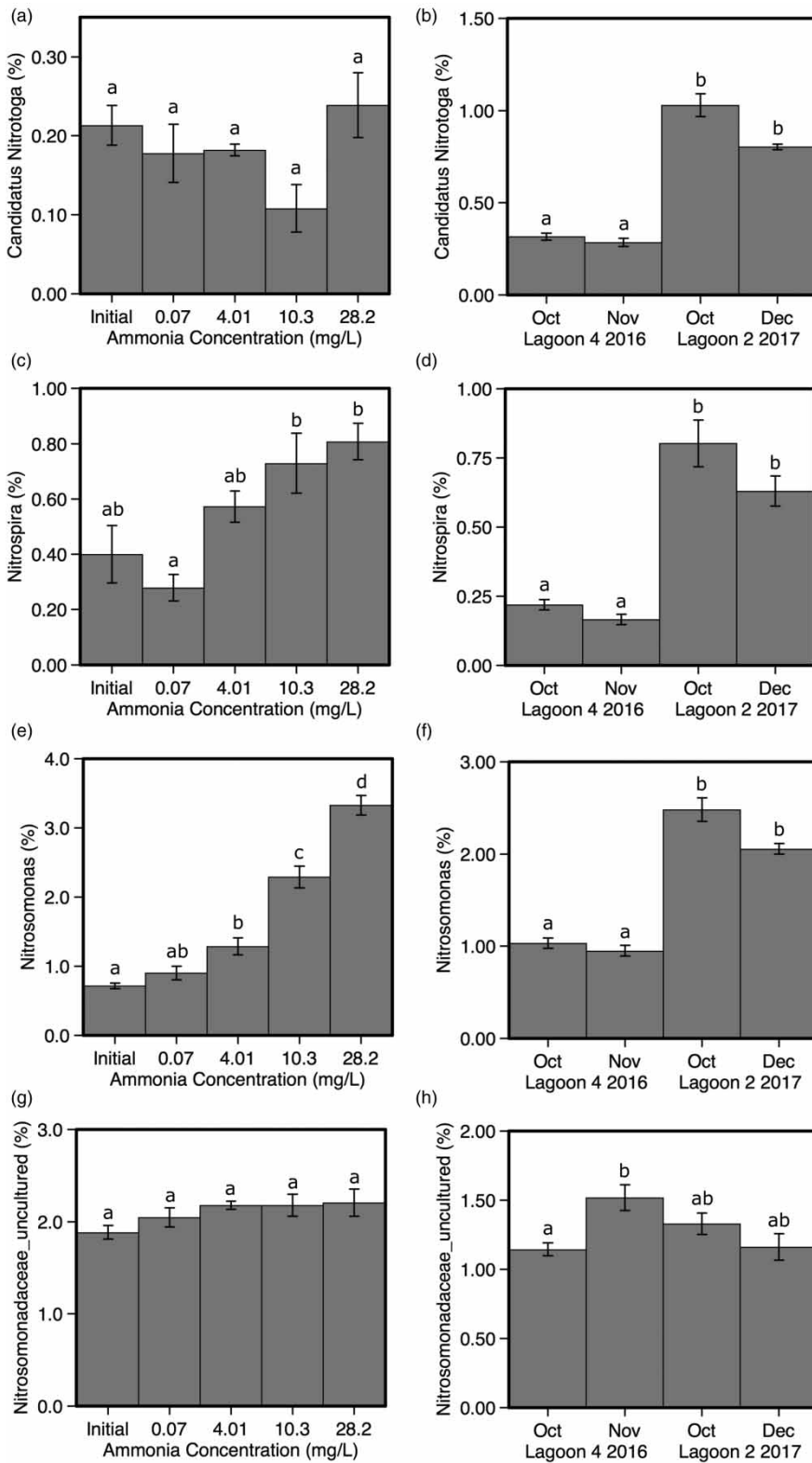


Figure 4 | Relative abundance of the identified nitrifying genera (mean \pm SE). (a), (c), (e), (g) The bench-scale samples with increasing ammonia concentrations (mg/L) of the inflow on the x-axis; (b), (d), (f), (h) the field samples. Letters above bars represent significant difference based on Tukey's post-hoc test ($p < 0.05$).

the growth of *Nitrospira* and *Nitrosomonas* on the BioCord substrate. The ratio of total AOB/total NOB ranged from 4.2 to 6.5. The optimal AOB/NOB ratio for nitrification was previously theoretically determined to be 2.0 with increased values representing potential anaerobic nitrogen removal (e.g. anammox) (Winkler et al. 2012). When the system was at its highest, $\text{NH}_3\text{-N}$ removal efficiency of $98 \pm 0.14\%$ with an inflow of 10.3 mg/L, the AOB/NOB ratio was 5.3, suggesting anaerobic nitrogen removal was an important metabolic process in treatment. This ratio did not significantly change when the removal efficiency dropped to $43 \pm 2.8\%$, suggesting the reduction in removal efficiency was not related to a specific microbial shift and that the treatment was potentially limited by residence time (Ødegaard 2006).

Nitrifying bacterial community from BioCord in field trials

Based on the results from the bench-scale experiments it was expected that *Nitrospira* and *Nitrosomonas* would be the respectively dominant NOB and AOB and would increase in dominance with increasing $\text{NH}_3\text{-N}$ concentrations. The BioCord bacterial community showed significant variation depending on where it was placed within the lagoon WWTP. This was shown by the large increase in relative abundance of *Candidatus Nitrotoga*, *Nitrosomonas*, and *Nitrospira* in L2 compared to L4 (Figure 4). Samples collected from L2 in 2017 showed an increase of total bacterial nitrifier relative abundance from 2.7% and 2.9% to 5.6% and 4.7% compared to the corresponding samples collected from L4 in 2016. The primary difference between bench-scale and field samples was that *Candidatus Nitrotoga* was the dominant NOB in the field while *Nitrospira* was dominant in bench-scale. Within the samples from both lagoons, *Candidatus Nitrotoga* and *Nitrospira* both showed no difference within the same lagoon but were both significantly more abundant in L2 ($p < 0.001$). *Nitrosomonas* similarly showed no differences within lagoons but was significantly more abundant in L2 ($p < 0.001$). In comparison, the uncultured genus of *Nitrosomonadaceae* maintained a relatively constant abundance between lagoons but showed an increase from October to November in L4 ($p = 0.002$). Although a relative abundance decrease in nitrifiers was expected with colder temperatures, there was no significant decrease of nitrifying genera observed in either lagoon. A previous study has shown that a reduction in temperature caused significant shifts in the species level of nitrifiers in wastewater treatment (Alawi et al. 2009). However, our study did not have species level

taxonomic resolution which may have identified potential relative seasonal differences.

In summary, the nitrifying bacterial population was more dominant in the BioCord community from L2. In contrast to the bench-scale study, *Candidatus Nitrotoga* was significantly more abundant than *Nitrospira* in the field samples suggesting that it could be favored when in a system subjected to day and night cycles and variable temperature conditions in the lagoon WWTP. A recent study determined that a species of *Candidatus Nitrotoga* carried genes encoding sulfite and hydrogen oxidation pathways allowing the organism to survive and proliferate during nitrite depletion, making it suitable for dynamic environments (Schwarz et al. 2018). Previous research has also shown that *Candidatus Nitrotoga* is the most well adapted NOB to colder temperatures which could explain its dominance in the temperate environment (Alawi et al. 2007). Although the taxonomy data from HTS quantified changes in the relative abundance of nitrifiers in the systems and showed no significant relative decrease in nitrifiers with colder temperatures, the results from RT-qPCR compared the absolute abundance values.

Variability in the abundance of key genes for nitrogen treatment from RT-qPCR

Nitrogen metabolic trends within the bench-scale experiment

Based on the results of the HTS data from the bench-scale study, primers were selected to quantify *Nitrospira*, *Nitrosomonas*, and anammox bacteria denoted by NOB, AOB, and AMX, respectively. All primers were selected through literature review and shown in Table A3. Representative genes responsible for denitrification were also quantified in both the bench-scale and field systems but were only confidently detected in the field system. The *Planctomycetes* bacteria targeted by AMX were not specifically determined with confidence at the genus level within the HTS data. However, there was a significant amount of uncultured OTUs from the *Planctomycetes* phylum seen in the taxonomy results throughout all samples. Therefore, the AMX primer was chosen to determine if the underdefined bacterial groups contained an active anammox population. A recognized limitation of 16S based primers are that they cannot conclude with certainty the specific metabolic activity of the organism, but the primary objective of this study was to show how the active community varies with physicochemical changes.

The gene abundances for AMX, AOB, and NOB were all determined to be highly abundant throughout the bench-scale experiment (Figure 5). Although evidence for denitrification was not observed in the bench-scale samples, the AMX presence suggests that anaerobic nitrogen removal processes still play a significant role. The average gene abundances for both AMX and AOB increased gradually but did not show a significant difference due to the inherent variability between replicates. This signifies that the biofilm community varied throughout its location on the BioCord which has been a feature identified with biofilm in previous studies (Yuan *et al.* 2012). In contrast, NOB showed a particularly sensitive response by significantly increasing in abundance with each increase of $\text{NH}_3\text{-N}$ ($p < 0.05$). This resulted in a 12-fold NOB increase from lowest to highest $\text{NH}_3\text{-N}$ influent concentration. When the influent $\text{NH}_3\text{-N}$ increased from 4.01 to 10.3 mg/L the removal efficiency remained elevated at $98 \pm 0.14\%$ even though there was only an observed increase in gene abundance for NOB. It is therefore likely that the increasing *Nitrospira* gene abundance contained significant comammox species. A similar trend was observed when $\text{NH}_3\text{-N}$ inflow was increased from 10.3 to 28.2 mg/L where NOB gene abundance increased two-fold.

The RT-qPCR gene abundance data for AOB, NOB, and AMX were compared with NNR to quantitatively determine which microbial groups were the strongest predictors of $\text{NH}_3\text{-N}$ removal in the BioCord system (Figure 5(j)). The gene abundance of AMX did not show any significant trends with NNR suggesting that most of the ammonia removed was due to nitrification, which was expected in the well oxygenated system. In comparison, there was a strong exponential relationship between NNR and both AOB ($R^2 = 0.9088$, $p = 0.0467$) and NOB ($R^2 = 0.9609$, $p = 0.0197$). This demonstrates that an increase in *Nitrosomonas* (AOB) and *Nitrospira* (NOB) significantly increased the NNR, with the latter showing the most significant relationship. This suggests that an increase in $\text{NH}_3\text{-N}$ concentration results in an exponential increase of NOB and AOB gene abundance in the BioCord, which results in an increase of NNR.

Nitrogen metabolic trends in the field trials

The elevated ammonia concentrations observed in L2 resulted in a significant increase of NOB gene abundance on the BioCord ($p < 0.001$; Figure 5). In comparison, there was not any significant difference in AOB when comparing samples within lagoons or corresponding samples between

lagoons. When the temperature decreased, there was a seven- and six-fold respective decrease of NOB in L2 and L4, but only the decrease in L4 was statistically significant ($p = 0.008$). Previous research also shows nitrification activity is inhibited by cold temperatures, which coincides with the large reduction of NOB (Ducey *et al.* 2010). Anammox bacteria were also found to be important in BioCord from both lagoons but did not decrease significantly within each lagoon as temperatures decreased. However, there were two times fewer AMX genes in L2 compared to L4 in the colder seasonal samples suggesting a reduction in anammox potential in L2 with decreasing temperatures ($p = 0.041$). Despite L2 having lower AMX gene abundance, its potential nitrogen removal was likely greater than L4 due to its significantly increased denitrification gene expression (Figure 5). Based on qualitative observation, the field BioCord samples had significantly increased biofilm thickness compared to the bench-scale samples. This would result in amplified zoning and layering within the biofilm which could account for the observed denitrification gene expression in lagoon samples. In the field BioCord, denitrification gene expression was measurable for all targets except *norB*, which was not measurable in L4. L2 showed relatively low but measurable levels of *norB* and significantly increased expression of *nirK* and *nosZ* ($p < 0.001$). There was no significant difference between samples within lagoons for all three denitrification genes. The elevated levels of *nirK* and *nosZ* genes in L2 specifically suggest that the bacterial community was removing more nitrogen from the system by metabolically producing more nitric oxide (NO) and nitrogen gas (N_2) (Weisener *et al.* 2017). Since the BioCord in L2 consistently had a larger population of nitrifying bacteria and increased denitrification gene expression, L2 maintained a higher nitrogen removal potential.

Chemical and microbial trends in Dundalk field trials

The collected data for the 18 chemical parameters along with the active taxonomy were analyzed using CCA to determine the potential chemical influence on observed microbial community differences (Figure 6). In general, the communities do not show significant variation within the same lagoon between October and December 2017 and October and November 2016. However, the communities vary significantly as the BioCord changes locations within the lagoon system from L4 to L2. The separation of the lagoons was primarily relative to the x-axis where L4 samples had positive x-values while L2 was negative. All six of the measured

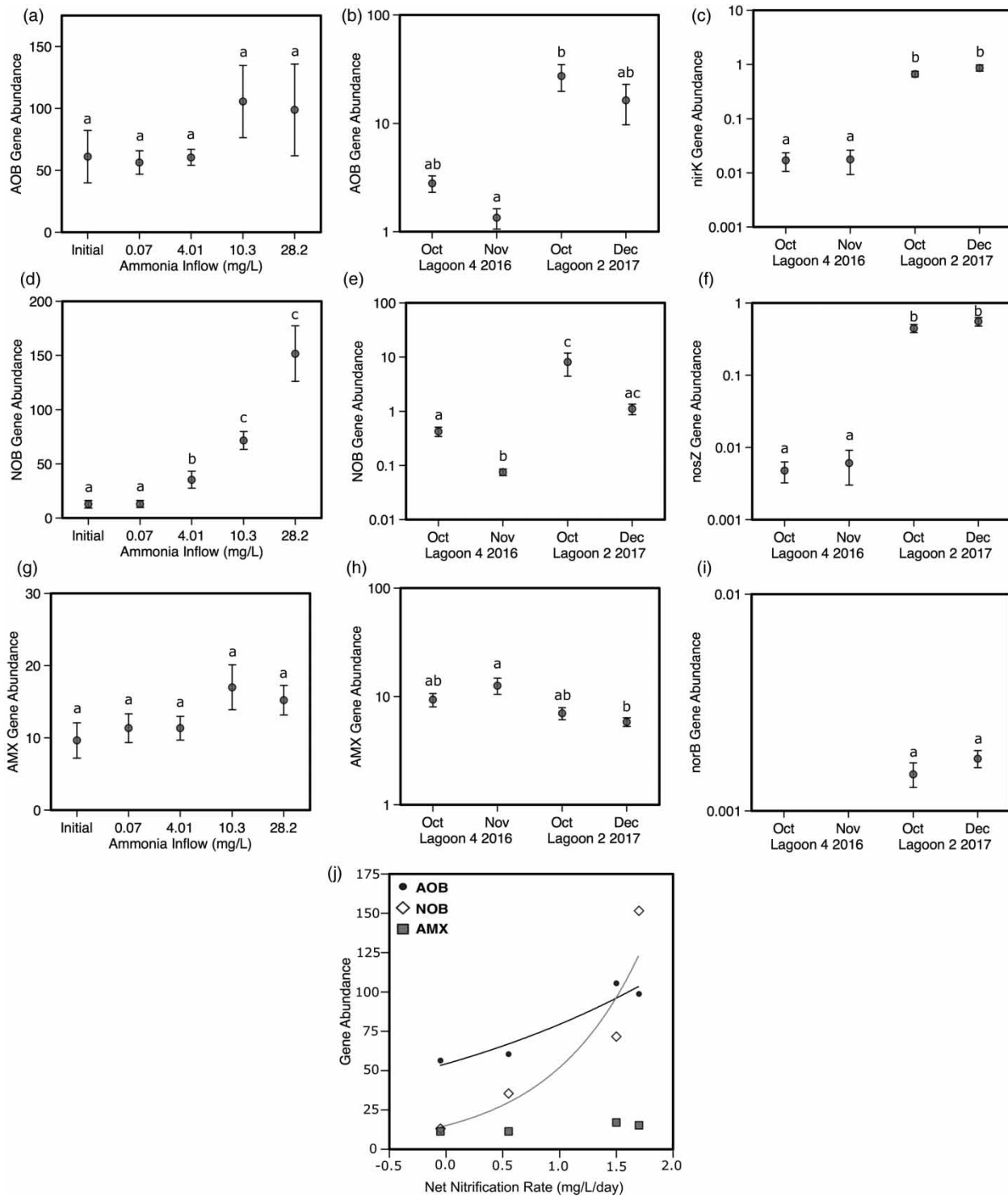


Figure 5 | Comparing gene abundance ($=N_0$ Target/ N_0 Control) for key genes (mean \pm SE). (a), (d), (g) AOB, NOB, and AMX in bench-scale samples with increasing concentrations of ammonia (mg/L) in the inflow; (b), (e), (h) AOB, NOB, and AMX in the field samples; (c), (f), (i) The gene expression of key denitrification genes nirK, nosZ, and norB in the field samples; (j) AOB, NOB, and AMX from the BioCord versus nitrification rates (mg/L/day) in the BioCord bench-scale system (AOB: $R^2 = 0.9088$, $p = 0.0467$ and NOB: $R^2 = 0.9609$, $p = 0.0197$).

nitrogen species are strongly negatively correlated with the x-axis (ranging from -0.792 to -0.978), representing the average five-fold decrease of TN from L2 to L4. This suggests

that an increase in nitrogen was driving the microbial community shift, which has previously been observed to be a major cause of community changes (Zhang *et al.* 2013;

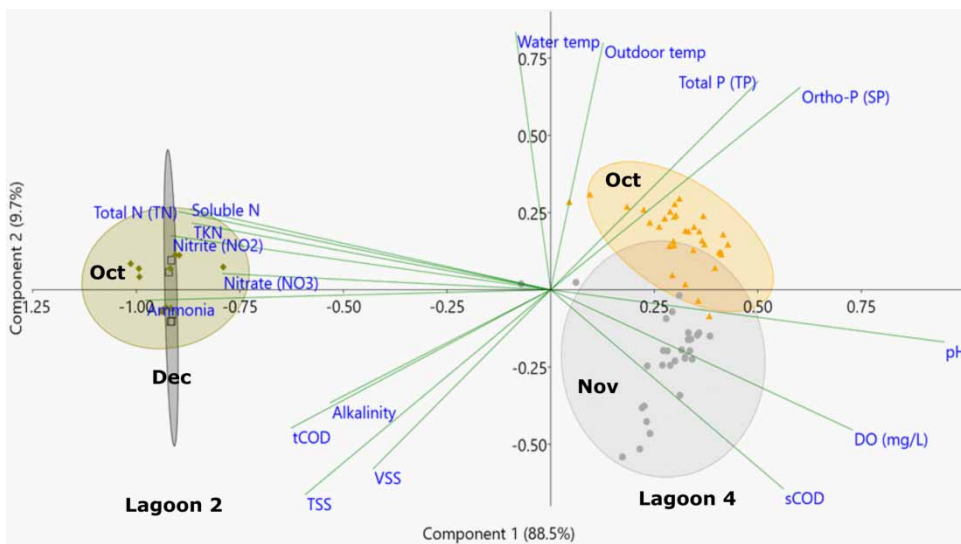


Figure 6 | Species conditional triplot based on canonical correspondence analysis of lagoon water chemistry parameters and OTU counts. The points represent the active community information based on OTU count from all the BioCord samples from the four sampling times. The 95% confidence ellipses each represent the grouping of samples from one of the four time points. Most of the variance was represented by the x-axis with 88.5% while the y-axis represents 9.7%. The measured quantitative chemical parameters are each shown by a green line. Please refer to the online version of this paper to see this figure in color: <http://dx.doi.org/10.2166/wst.2020.170>.

Weisener *et al.* 2017). This corresponds with the observed increase in nitrifier abundance and denitrification gene expression in L2.

The pH has a strong positive correlation with the x-axis (0.950) suggesting that the elevated pH in L4 contributed to community changes. Microorganisms have an optimal pH range for growth and thus wastewater pH has been shown to greatly influence the microbial community in WWTP (Gao *et al.* 2016). Denitrifiers and nitrifiers have previously shown optimal pH ranges from 7.0 to 8.0 and 6.5 to 9.0, respectively (Pan *et al.* 2012; Zhang *et al.* 2012). The elevated pH in L4 (8.3 and 8.4) could therefore have contributed to the significantly decreased denitrification gene expression.

The greatest separation between samples within each lagoon was based on the y-axis with which water temperature was the most strongly correlated parameter (0.834). Temperature is known to significantly change microbial metabolism and therefore influence wastewater treatment (Chen *et al.* 2017). This suggests that temperature was the primary driver between the sampling points within each lagoon, which only resulted in significant differences between L4 samples. However, the temperature decrease (16.9 to 1.6 °C) in L4 did not cause the total relative abundance of total observed nitrifiers to change. This supports previous chemical observations that nitrification performance is less effected by low temperature in attached biofilm systems (Xing *et al.* 2013). The combined chemical and microbial results from this study provide important information to

understand the bacterial community dynamics of BioCord in wastewater treatment to better predict and monitor changes with variable chemical and seasonal conditions.

CONCLUSIONS

High-throughput sequencing and RT-qPCR showed that the abundance of nitrifiers significantly increased with an increasing $\text{NH}_3\text{-N}$ gradient, resulting in up to $98 \pm 0.14\%$ $\text{NH}_3\text{-N}$ removal in bench-scale experiments. Based on stable isotope analysis, $^{15}\epsilon$ and $\delta^{18}\text{O}_{\text{NO}_3}$ values were effective regulatory monitors of enhanced biological nitrification rates. The BioCord bioreactors in the lagoon WWTP showed a significant response to the changing nitrogen gradient and contained more nitrifiers and increased expression of denitrification (norB, nirK, and nosZ). Anammox bacteria were active community members within the bench-scale and field BioCord. At colder temperatures (2 °C), the relative abundance of total active nitrifiers on the BioCord was maintained.

ACKNOWLEDGEMENTS

We express thanks to funding support for this project from MITACS (1T08131), Bishop Water Technologies (33062), Ontario Genomics (33336), and the NSERC

ENGAGE plus program. Additional support was provided by NSERC's Discovery Grant program (SOCM and CW). We would also like to thank Thomas Reid and Sara Butler for their suggestions and assistance throughout this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at <https://dx.doi.org/10.2166/wst.2020.170>.

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First received 8 October 2019; accepted in revised form 31 March 2020. Available online 10 April 2020